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ISOLATING A CELL MAXIMALLY SECRETING ACETYLCHOLINESTERASE

FINAL REPORT

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Techniques are described for potential selection of overproducers of secreted protein such as acetylcholinesterase. In addition, large scale cell culture of a rodent hybridoma cell line has resulted in the production of milligram quantities of purified enzyme.

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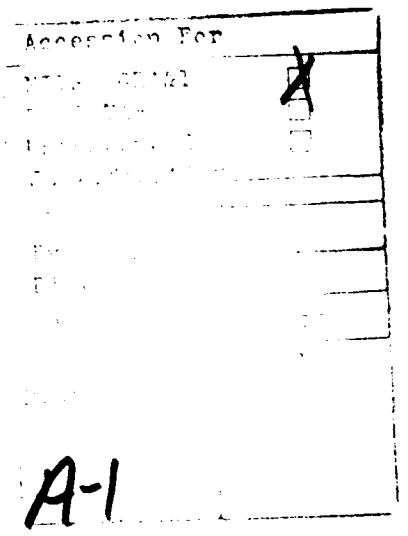
SUMMARY

Progress has been made in developing cell-sorting techniques for selecting cells which secrete certain proteins of interest such as acetylcholinesterase. Model systems using hybridoma cells have indicated that the fluorescence activated cell sorter (FACS) may be used to accomplish this goal. Specifically, antibody-secreting cells were enriched using this technique from a larger population of non-secreting cells. However, this technique could not be developed sufficiently during the contract period to be used to isolate cells secreting acetylcholinesterase.

Project emphasis was shifted after the first year to continued development of the cell-sorting approach and simultaneous provision of up to 100 mg of acetylcholinesterase from a cell line to be provided by USAMRDC. After approximately 60 days of growth in our large-scale cell culture system, we were able to process 15 mg of product from approximately 2600 liters of conditioned medium. The final product contained approximately 5 mg after purification.



FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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I. Summary of objectives. The overall objective of the contract was to conduct a research program designed to develop a stable, transformed cell line which could be grown in mass culture and which is capable of producing a minimum of 1% of its total protein as human acetylcholinesterase (AChE). More specific milestones in achieving these goals were as follows:

1. Cell line selection. From up to 5 cell lines, one cell line was to have been chosen for further work based on suitable growth characteristics and AChE secretion.

2. Development of two selection systems for AChE overproduction. Two systems each capable of selecting cells based on the secretion of AChE were to have been developed. One system was to have been based on a density shift technique whereas a second system was to utilize a fluorescence-activated cell sorter (FACS).

3. Enhancement of production via gene duplication. Using technologies described in the preceding section (2), a stable, transformed cell line capable of producing up to 1% of its total protein as AChE was to have been developed. Overproduction per se (as opposed to selection for overproduction) was to have been achieved through spontaneous gene duplication events, following treatment of cells with agents such as hydroxyurea which enhance gene duplication events, or as a result of the transfection of genomic DNA from cells which secrete AChE.

4. Molecular cloning of the AChE gene. From overproducing cell lines which had been developed as described in the previous section (3), the AChE gene was to have been isolated by molecular cloning.

5. Provision of AChE. At the end of the contract, at least 100 milligrams of human AChE was to have been delivered. This objective was modified February 9, 1987 such that we were obligated to provide at least 6 milligrams of mammalian AChE.

II. Specific achievements over the contract period.

1. Cell line selection. Cells of either neuronal or neuromuscular origins were tested because of the

association of AChE with these tissues. Of the 5 cell lines tested, the human rhabdomyosarcoma A204 secreted the most AChE on a per cell basis. To the best of our knowledge, this cell line has never been characterized with respect to AChE secretion. Levels of production were, however, 5-10-fold lower than that of the mouse neuroblastoma cell line NB41A3 which had been characterized for AChE secretion. An important observation made was that filtration of either bovine serum or lymph through 100,000 Kd molecular weight cut off filters essentially eliminated background AChE activity in growth medium. This approach was subsequently employed by members of Dr. Ken Hunter's group (In Vitro Cell. Dev. Biol. 22, 670, 1986).

2. Development of two selection systems for AChE overproduction. Because of the extremely low levels of AChE produced by the A-204 cells (e.g., approximately 20 picograms/10^6 cells/day), for the development of cell selection methods, model systems using higher producers were initially employed. In a second phase, the application of methods to AChE secretion was to be attempted. We were, however, successful in developing the basic method of cell selections for model systems only at the very end of the contract period. There was, therefore, insufficient time to pursue selection for AChE.

   a) Selection of secreted proteins by fluorescence activated cell sorting. (Cell Isolation Technique - CIT) The majority of the contract period concentrated on methods to encapsulate single cells within colloidal beads approximately 50 microns in diameter, the development of protein capture mechanisms which would prevent secreted proteins from "leaking" out of beads, the physical aspects of flow-sorting large, fluorescently labeled particles, and recovery and grow-up of cells selected by the process. Results showed that the technique was capable of selecting qualitatively for a minor population of antibody producing cells (hybridomas) from a 20-fold excess of non-producers. Secondly, we were able to select for higher producers of a tissue plasminogen activator (t-PA) from a human melanoma cell line (1.5 - 1.8-fold greater than control, unsorted cells).

b) Selection by density shift. This approach in which a secreted protein set in motion a complex series of events which ultimately resulted in decreased density of beads containing secretors was abandoned for reasons of non-reproducibility and lack of quantitative discrimination. Pilot experiments using hybridomas showed that the technique might be utilized in a qualitative sorting procedure, i.e., selection of productive hybridomas from non-productive cells.

Details of the above experiments are described below:

Cell encapsulation. Central to the success of the CIT approach is the encapsulation of single cells within colloidal beads less than 50 u in diameter. The small size is dictated by the fluid mechanics of the sorting apparatus of the FACS. The procedure used for cell encapsulation is described below:

PROCEDURE FOR CELL ENCAPSULATION IN AGAROSE BEADS

INGREDIENTS:

1. DOW Silicon Oil, Dimethylpolysiloxane (1000 cs)
2. 4% Agarose (Seaplaque low geling)
3. Pluronic F-68
4. DOW Silicon Oil, Dimethylpolysiloxane (5 cs)
5. Latex Beads (Interfacial Dynamics Corporation)
6. Coupled Antibody

EQUIPMENT:

1. Graduate cylinder or other suitable volume measuring device

2. Polypropylene tubes (4 ml) Falcon tubes (15 and 50 ml)

3. Balance - 30 g capacity

4. Vibromixer

5. Waterbaths (45 degree and 0 degree)

SUPPLIES:

1. pH calibration solutions, 4 and 7

2. Pipettes, graduates, beakers and Tissue Culture Water (TCW) filled wash bottles as needed

PROCEDURE:

A. Encapsulation of Cells

Place 25ml of 500cs oil (DOW silicon, dimethylpolysiloxane 1:1 mixture of 1000 cs and 5 cs) in a 50 ml Falcon tube and place in 45°C water bath. Place a tube of 4% Agarose in saline (FMC, Seaplaque low geling) in 70°C water until it melts, then place it also in the 45°C water bath. Place 1-3 x 10^7 cells suspended in 900 ul of PBS in a 4 ml polypropylene tube and add 20 ul of F-68 pluronic (BASF, 10 mg/ml in saline), invert the tube to mix, and place it in the 44° ±2°C water bath. Next add 1 ml of the agarose to the PBS/Pluronic and vortex mix while keeping tube warm. Pour the warm oil into a 50 ml beaker set in the vibromixer jig. Turn the vibromixer power to 110 volts and add the agarose suspension drop-wise to the oil (taking no longer than 5 sec.) and allow the two phases to emulsify for 120 ± 5 sec.. Now add 25 ml of -20° ±5°C 5 cs oil (DOW silicon) while still mixing and place an ice bath around the emulsion beaker. Allow the emulsion to set-up for 5-10 min. Pour the emulsion back into the 50 ml Falcon tube and centrifuge (300g, 3 min.), pour off the oil phase and add 10 ml of PBS or MEM and vortex mix until the agarose beads are resuspended. Pour the agarose beads into a 15 ml falcon tube and centrifuge, decant off the overlaying oil and media. Using a new 15 ml tube resuspend the agarose beads in 10 ml of PBS or MEM and centrifuge. Pour off the overlaying media and finally resuspend beads in media.

B. Encapsulation of Cells and Latex

Place 25 ml of 500 cs oil (DOW silicon, dimethylpolysiloxane) in a 50 ml Falcon tube and place in 45°C water bath. Place a tube containing 1 ml of 3% Agarose (FMC, Seaplaque low geling) in 0.3M Sucrose (isotonic) pH 7.0 buffered with 4mM phosphate in 70°C water until it melts, then place it also in the 45°C water bath, add 100 ul of Pluronic F-68 solution (1% w/v in deionized water).

Prepare a cell suspension as in procedure A and mix with 400 ul latex suspension and warm to 37°C. Next pipette the cell/latex suspension into the agarose and vortex mix. The emulsion oil is now poured into the vibromixer jig and agitation is started. The agarose/cell suspension is next poured into the emulsion oil and vibromixer power brought up to 110 volts. Emulsify for 2 minutes than add 20 ml of -20°C, 100cs oil and continue to mix for 5 seconds. Stop

agitation and allow emulsion to set in ice bath for 20 minutes. Pour the emulsion over 10 ml of MEM in 50 ml centrifuge tube and centrifuge for 5 minutes at 350g. Decant the oil phase and wash the resulting beads once more in MEM.

C. Encapsulation of Cells and Coupled Antibody

As in procedure A except that 1 mg of dextran antibody conjugate in 200 ul PBS is added to the 9[illegible] ul PBS cell suspension and this mixture is encapsulated.

While considerable efforts were expended on this aspect durin~ the first year of the contract, reproducibility and recovery of cells continued to plague the process. As a result of more than 30 experiments during year two of the project, the process has been brought under control. It is now possible to achieve over 80% encapsulation and recovery of cells in beads under 100 u in diameter (Figure 1A).

Insolubilization of trapping antibody. In order to prevent secreted cell products from leeching out of the agarose beads, a trapping antibody, specific for the secreted product such as acetylcholinesterase, must be immobilized within the beads containing cells.

Initial experiments attempted to utilize colloidal gold particles 20-30 nM in diameter to which antibody or other protein spontaneously adheres. Because of the irreproducibility of the technique and the difficulty of physically handling these particles, this approach was abandoned in favor of carboxylated latex spheres, 1 u in diameter, to which antibody was covalently coupled using the technique of Molday et al (1). After coupling, the derivatized latex spheres were characterized for antigen binding capacity at saturation.

In practice, cells along with these derivatized particles are coencapsulated within the bead. This results in a bead with, on average, 1 cell and about 50 particles. These particles do not

appreciably contribute to the light scattering or alter the physical characteristics of the bead itself. Representative beads with cells and fluorescein-labeled latex particles are shown in Figure 1B.

Model studies. In order to establish the basic parameters associated with this approach to CIT, two types of cells were initially employed in model studies. The first cell type chosen was a mouse hybridoma cell line which secreted a monoclonal IgG_1 to an unknown antigen. The secretion rate was approximately 1000 immunoglubular molecules/cell/second. Because of its high rate of secretion, this cell would, in theory, give the greatest signal to noise ratio during the actual sort. The second cell type investigated was a human melanoma cell line which secretes tissue plasminogen activator at a rate of approximately 10 molecules/cell/second. Thus, with a 100-fold lower secretion rate, this cell represents a significantly greater challenge and it approaches levels seen for acetylcholinesterase (AChE) secretion. Both cell lines were proprietary to Bio-Response.

Murine hybridoma. The goal of this model study was to determine whether the system was capable of identifying and physically isolating a hybridoma cell which secretes a monoclonal antibody from a significantly greater population of cells which do not secrete that product. During this phase we only wished to determine whether the physical aspects of sorting could be established; no actual expansion of the sorted population was to be attempted.

The mouse hybridoma cells were mixed with a human Jurkat cell at a ratio of 1:20, thus giving a population of which only 5% of the cells secreted a monoclonal antibody. In addition, the hybridoma population was prelabeled overnight with [^{14}C]-thymidine to enable identification of these cells by autoradiography both before and after the separation. To capture the secreted monoclonal antibody, carboxylated latex spheres (Polyscience, Ind., Warrington, PA) were derivatized with goat anti-mouse IgG (Cooper

Biomedical, Malvern, PA).

Following coencapsulation of the reconstructed population with derivatized particles, cells (i.e., beads) were incubated overnight in medium to allow for accumulation of antibody and absorption to the immobilized antibody. Next, cells (beads) were washed and incubated with fluorescein-labeled goat anti-mouse IgG and after 1 hour excess antibody washed free of the beads. The optimal amount of this second antibody used was determined in reconstruction studies (not shown). Finally, the population was interrogated by FACS. From the distribution of fluorescent beads shown in Figure 2, the uppermost fluorescent 5% of the population was sorted from the rest of the population. Cells from these selected beads were isolated, fixed on microscope slides, and processed for autoradiography using Kodak NTB-3 emulsion. After a suitable exposure period, the slides were developed and stained to reveal nuclei, and labeled nuclei were counted and compared to unlabeled nuclei in both the selected population and the starting, unsorted population. These data are displayed in Table 1. Whereas the presorted (starting) population was composed of, as planned, 5% antibody-secreting cells, this percentage was increased to 54% in the post-sort population. In other words, the sort resulted in a 10-fold enrichment of antibody-producing (i.e., ^{14}C-labeled) cells. We feel confident that the unlabeled cells present in this population could have been reduced still further if a greater degree of discrimination (i.e., 1% vs 5%) was employed. Nevertheless, this represents the first concrete demonstration that the approach actually works. These results were obtained in January 1986.

Human melanoma. Initially, a protocol virtually identical to that used in Model 1 was used and showed that the system could also enrich for cells which secrete the t-PA. Naturally, different antibody reagents were employed. Specifically, goat antiserum to the protein of interest was both immobilized to the latex

particles and fluoresceinated for use as a second, labeled antibody.

A second experiment was performed most recently in which the uppermost fluorescent 10% of encapsulated melanoma population was sorted, liberated from the beads, expanded for analysis, and resorted three times. These results showed a 1.8-fold increase in relevant protein secretion per cell after multiple sorts.

High producers of AChE. Because the CIT technology took significantly longer to develop than originally proposed, we were unable to adequately characterize the approach before the expiration date of the contract.

3. Enhancement of production of AChE via gene duplication. Since we could not develop the basic selection techniques for higher production of AChE within the time limits of the contract, no experiments directly leading to this end were attempted.

4. Molecular cloning of the AChE gene. The cloning of the AChE gene could only have been accomplished with the successful selection of an overproducer of AChE. Since this was not accomplished, no efforts were made in this area.

5. Production of AChE. Because it was clear after the first year of the contract that we most probably would not achieve our stated objectives, emphasis was shifted to the large scale growth of the E-2 cell line developed by Dr. Ken Hunter's group which produces a rodent (not human) AChE. The goal was to produce approximately 6 milligrams of purified material and provide this material to Dr. Jerry Sadoff, Department of Bacterial Diseases, Walter Reed Army Institute Research. After receiving the cell line during the second quarter of 1985, we determined that the E-2 cell line would best be grown in large scale in an anchorage dependent mode as opposed to in suspension. Further, a low protein, serum-free medium with which we had familiarity was found to be suitable for production periods of up to one week. If, however, the cultures were periodically pulsed

with serum, extended production in low protein medium could be continued for many months.

For large scale production of AChE, 5 bioreactors were established and operated in the serum pulse mode for about 6 weeks. Over that period, greater than 2600 liters of conditioned, serum-free medium were collected and concentrated. Thereafter, the material was affinity purified over procainamide Sepharose and desalted. The final material was made 50% in glycerol, and stored at -20°F. This material was prepared in February 1986, but was sent to Dr. Sadoff in April 1987 because of delays in modifying the specifications of our contract that would allow us to substitute "mammalian" AChE for "human" AChE.

We estimate that approximately 15 mgs of rodent AChE were produced. If this material had been produced in conventional roller bottle culture, over 5000 liters of conditioned medium would have been required at a cost of approximately $50/liter. After purification, approximately 4.7 milligrams remained as a result of recovery of about 25%.

Further details concerning the production of AChE are contained in Section IV of the July 7, 1986 Annual Report and Reference 1.

6. Characterization of the final product. The specificity of the final product was established through the use of specific (BW284C51) and nonspecific tetraisopropyl pyrophosphoramide (ISO-OMPA) inhibitors of AChE both in the radiometric assay and in fluorescence zymography of samples electrophoresed through 6% native polyacrylamide gels (Dr. R. Schuman, personal communication). In Figure 3A, the specific identity of final product as AChE is established by this approximate 4 log shift in I_{50} values when enzyme assay were performed in the presence of varying concentrations of either inhibitor. Of interest is the enhanced sensitivity of rodent AChE to the specific inhibitor when compared to the enzyme from chicken (I_{50} - 1 x 10^{-8} vs 5 x 10^{-6}M) (5). For comparison, in Figure 3B similar inhibitor studies were performed with human AChE obtained from red blood cells (Sigma).

As an independent confirmation of specificity and in

an attempt to gain insights into the structure of the enzyme, various samples were electrophoresed through 6% native polyacrylamide gels and stained for esterase activity using the fluorometric substrate N-methyl indoxyl ester (Dr. Schuman, personal communication) (6). Figure 4 shows three gels loaded and run identically and in parallel. Lane 5 with purified final product shows a major and minor esterase band, both of which are extinguished by the specific inhibitor (gel B) but not the nonspecific inhibitor at a 100-fold greater concentration. This behavior is shared with the human AChE (lane 4) but is different from horse serum butyrylcholinesterase (lane 3). Not visible in the gel reproductions are faintly staining bands in NCS lanes (lane 2) which appear to be specific for AChE and completely disappear upon heat treatment (lane 1). This latter result confirms data presented in Figure 5. Finally, none of these data indicate probably form or molecular weight of the final product although Rick Schuman, a co-developer of the cell line along with Dr. Ken Hunter, believed the major form to be a G_4 structure of about 330,000 daltons.

A final experiment to detect possible contamination of concentrated product with bovine AChE was performed. A monoclonal antibody, AE-1, reacts with bovine and human forms of AChE, but not the rat or mouse species of protein (Figure 6). The final product was treated according to the protocol in Appendix 3.

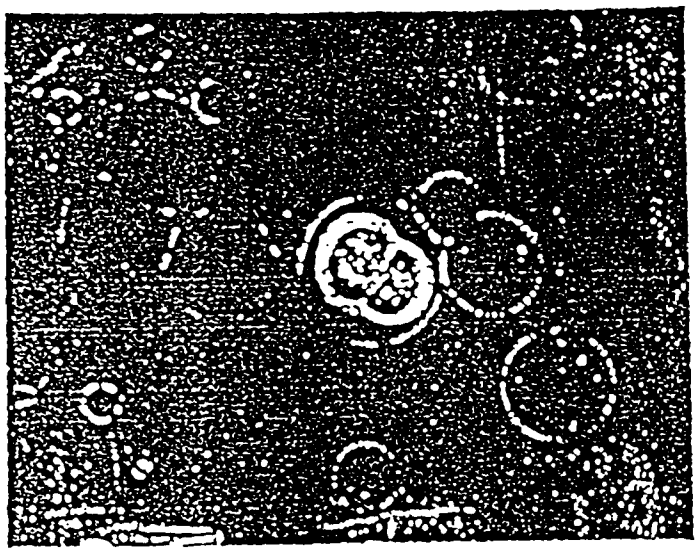
Bovine serum AChE was detectable, but no activity could be obtained from the final product. The major and minor bands obtained after gel electrophoresis and reaction with N-methyl indoxyl acetate are not due to the presence of active bovine AChE.

7. Disposition of final product. The purified material was sent to Col. Jerry Sadoff, Department of Bacterial Disease, Walter Reed Army Institute of Research, Building 40 - Room 2085, Washington, D.C. 20307.

REFERENCES

1. Brown, P.C.; Figueroa, C.; Costello, M.A.C.; Oakley, R.; and Maciukas, S.M. (1988) Protein Production from Mammalian Cells Grown on Glass Beads, Animal Cell Biotechnology 3, 252-262.

A.



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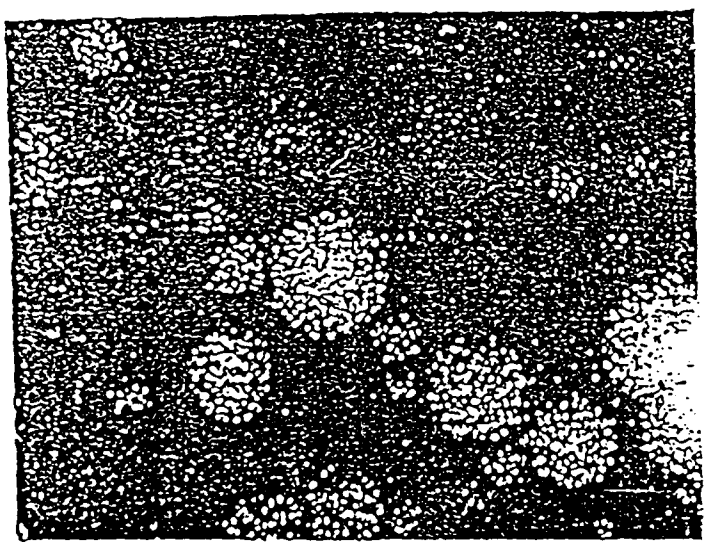

Figure 1. Encapsulation of cells without (1A) and with fluoresceinated latex particles (1B). Cells were encapsulated as indicated in the text.

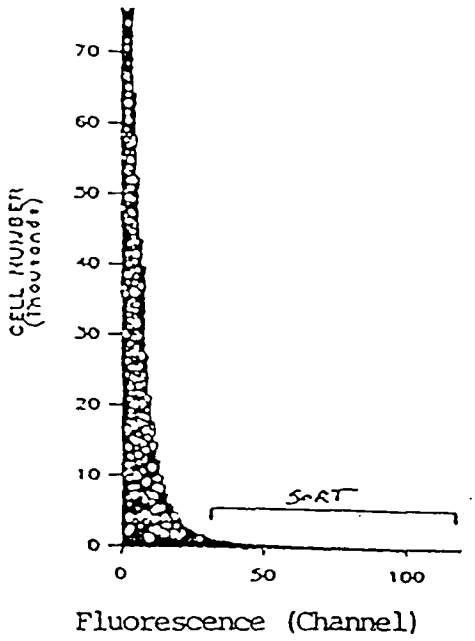

Figure 2. Model System 1: fluorescence distribution and sort criteria. A 1:20 mixture of 8501 murine hybridoma cells and human Jurkat cells was encapsulated and sorted using the FACS instrument. Channels 27-124 which comprised the uppermost fluorescent 5% of the population were sorted and processed for autoradiography (see Table 1).

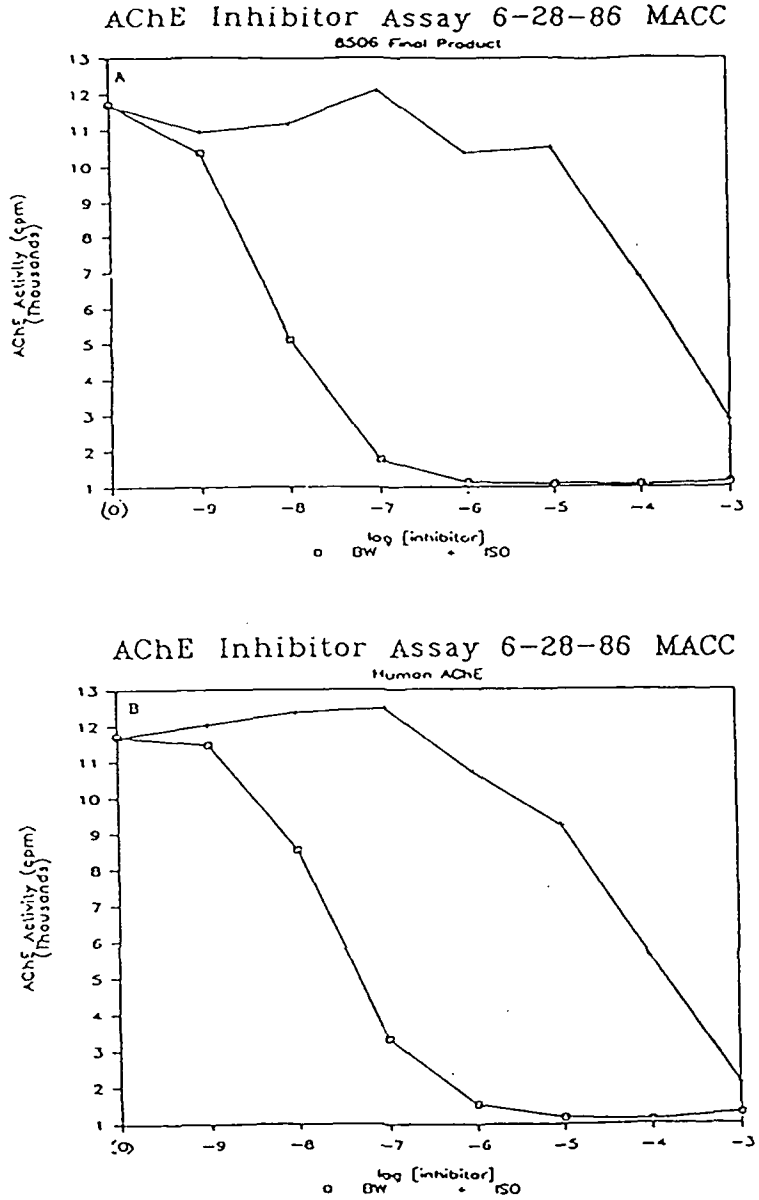

Figure 3. Effects of specific and nonspecific AChE inhibitors. A, samples of purified final product were assayed for AChE activity in the radiometric assay in the presence of varying concentrations of specific inhibitor (BW284C51) and nonspecific inhibitor (ISO-OMPA). The I_{50} values for the specific inhibitor were approximately 1 X 10^{-8}M; for the nonspecific inhibitor the corresponding value was approximately 1 X 10^{-4}M. B, radiometric assays were performed with commercially available human AChE (Sigma).

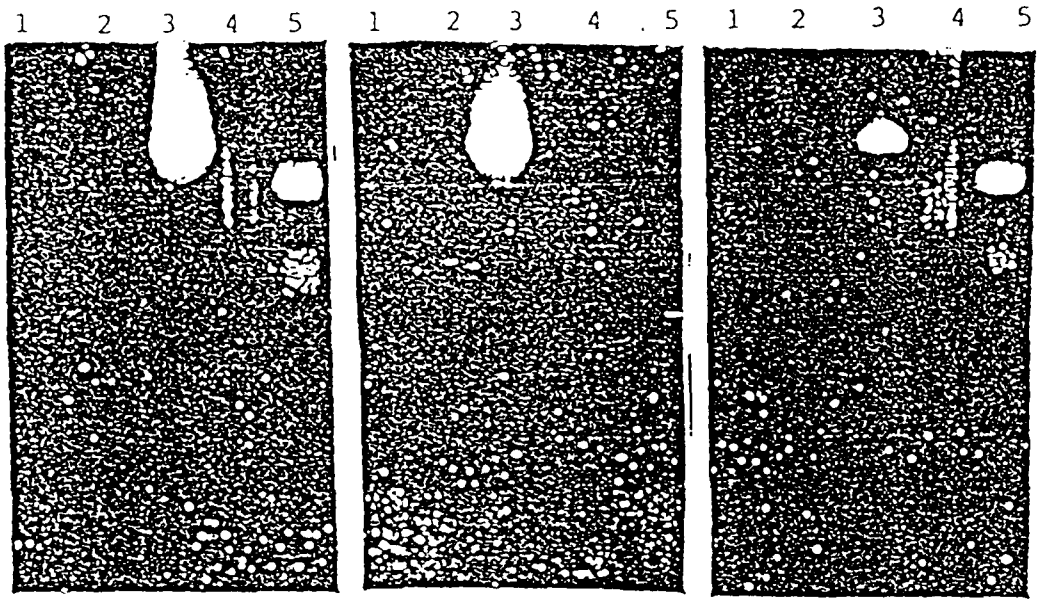

Figure 4. Identification of AChE activity by electrophoresis and fluorescence zymography. Various samples were electrophoresed through 6% polyacrylamide gels in the absence of sodium dodecyl sulfate (SDS) and reducing agent in TRIS-glycine buffer, pH 8.3. Subsequently, the gels were stained with N-methyl indoxyl acetate (2×10^{-3}M) for 30 minutes at 37°C in the dark, transilluminated with a long-wave ultraviolet light source, and photographed. Lane 1, heat-treated newborn calf serum, 15 ul; lane 2, newborn calf serum, 15 ul; lane 3, serum butyrylcholinesterase, 15 IU; lane 4, human red blood cell AChE, 40 IU; lane 5, purified final product, 15 IU. Gel A, native; gel B, preincubated in BW284C51 (1×10^{-7}M); gel C, preincubated in ISO-OMPA (1×10^{-5}M).

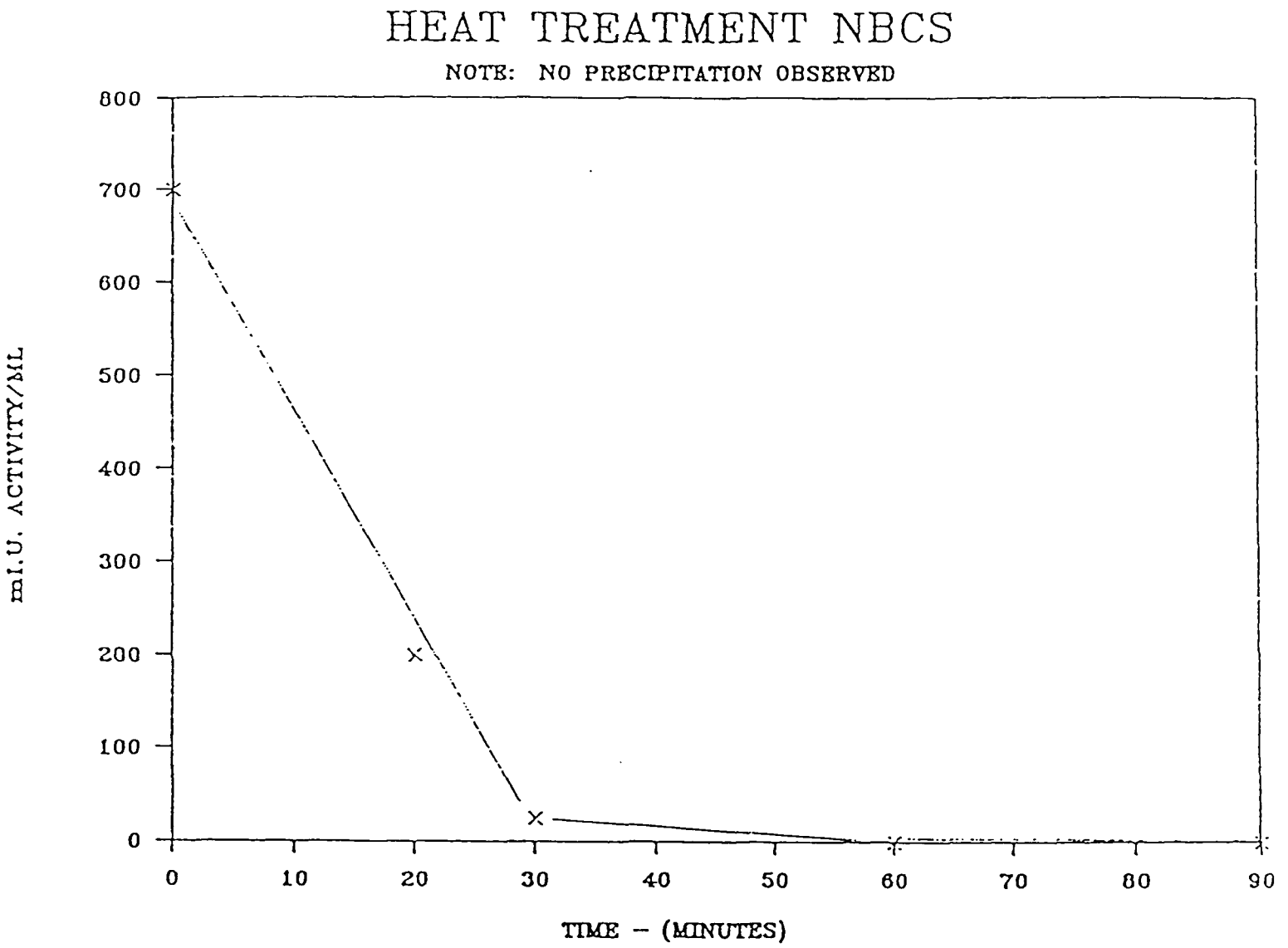

Figure 5. Heat treatment of NBCS. Aliquots of NBCS were incubated for varying lengths of time at 56°C. AChE assays (radiometric) were performed on each aliquot at the end of 90 minutes.

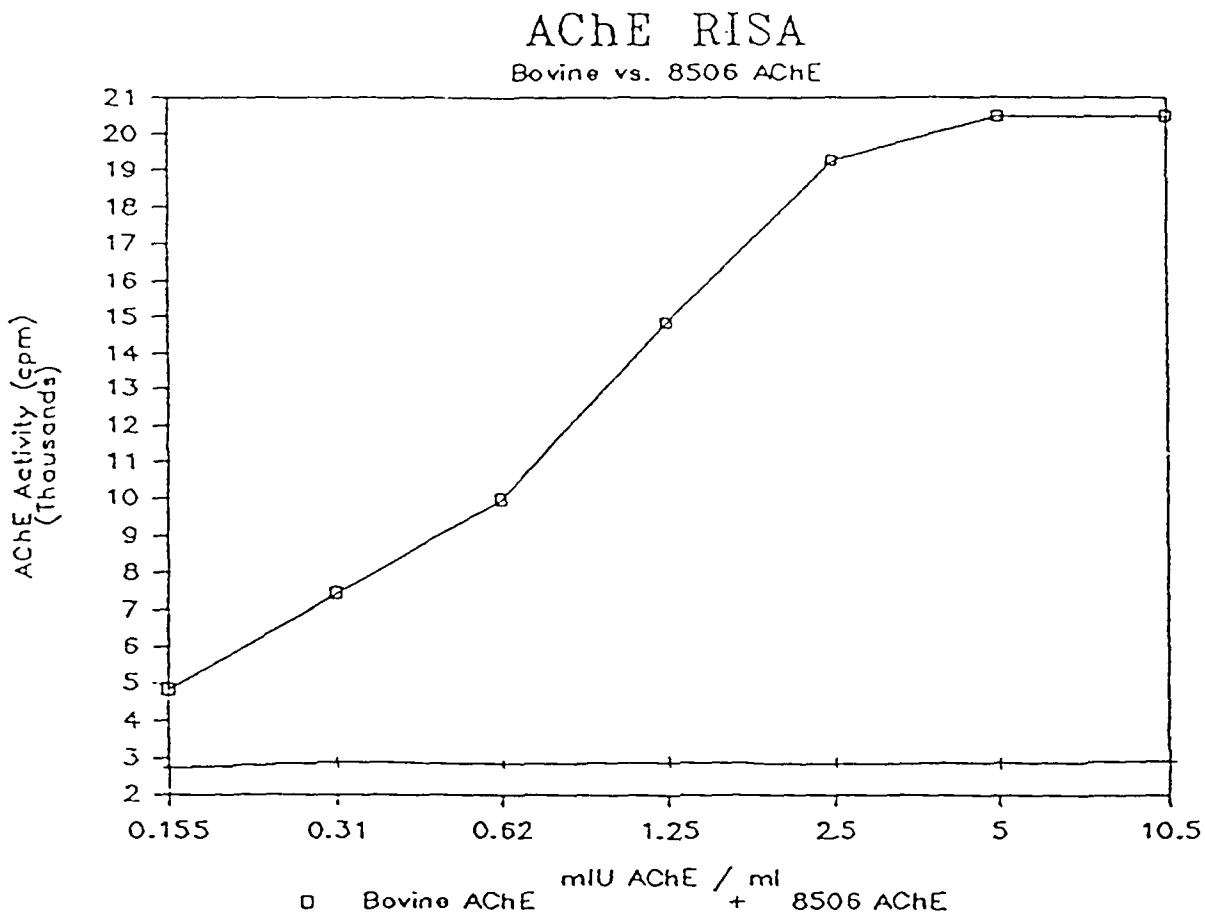

Figure 6. Reactivity of final product in radiometric immunoadsorbent solid-phase assay (RISA). AE-1-monoclonal antibody recognized both human and bovine AChE but not the rat or mouse species of AChE (data not shown). Samples for final radiometric assay were preincubated with the AE-1-antibody to isolate activities due to bovine AChE. A standard prepared with bovine serum was tested in comparison to final product.

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